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Molecular MR Imaging of Protease Activity in Breast Cancer with Activated Contrast Agents

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14. ABSTRACT

We synthesized and characterized a smart MR contrast agent activated by Cathepsin-D enzyme. The agent was tested in a cell free system and in vitro with immobilized breast cancer cells. We also initiated animal studies however due to the finding that the structure of the probe needs to be optimized we are currently refining the agent for in vivo applications.

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Introduction

Contrast agents activated by biological processes can be extremely useful for functional imaging of various diseases including cancer, which is typically characterized by unique microenvironment with increased concentration of proteolytic enzymes, hypoxia, and acidosis. This concept has been successfully applied for NIR optical imaging, however it remains an elusive technology for MRI, although several methods had recently been explored including activated chelating compounds for Gd and clustering of SPIO nanoparticles to increase T2 relaxivity. Here, we report a novel class of activated contrast agent for MR imaging developed using combination of superparamegnetic iron-oxide nanoparticles (SPIO) with multiple Gd groups. This novel technology is called dual contrast technique. When both components are localized in close proximity the resulting complex will produce strong negative T2 contrast in T1/T2-weighted MRI due to high T2 relaxivity of SPIO particles. Upon dissociation of the complex, small Gd groups will diffuse away from the SPIO core and generate positive T1 MR contrast. In this project, we focus on Cathepsin-D that is a proteolytic enzyme overexpressed in breast cancer cells, and MR contrast agent can be activated by this enzyme in acidic extracellular tumor microenvironment. We are presenting our initial data obtained in vitro and currently are working on in vivo applications of this new technology.

Body

We completed synthesis of the activated MR contrast agent using three component system: streptavidin-coated SPIO nanoparticles, and functionalized gadolinium chelates. The agent was successfully tested in cell-free systems and in vitro with breast cancer cells. Limited activation of the agent by breast cancer cells possibly due to the inhibiting action of serum suggests that the structure of the cleavable linker needs to be optimized to render it feasible for in vivo applications.

1. Months 1 – 6.

To synthesized and characterize the activated MR imaging agent using an SPIO core, Cathepsin D specific cleavable peptide linkers, and gadolinium chelates. For the prove of the principle studies a model system consisting of streptavidin-functionalized SPIOs and biotinylated peptide linkers conjugated to bifunctional DTPA ligands and FITC fluorescent probes will be synthesized.

The contrast agent was synthesized using 50 nm streptavidin-coated SPIO nanoparticles (Miltenyi Biotech Inc.) conjugated with Cathepsin D (CaD) substrate peptide, biotin – GGPI-C(Et)-FFRLGGK-OH. Free anime of the terminal lysine was reacted with GdDOTA-NHS ester (Macrocyclics, Dallas, TX) with a conjugation ratio of 2-6•10^5 gadoliniums per SPIO determined by ICP-MS. The average nanoparticle diameter determined with DLS was about 92 nm. The chemical structure and mechanism of activation of the agent are shown in the Figure 1.

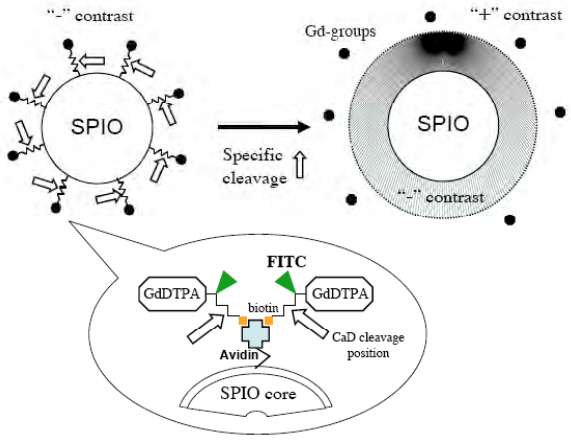

Figure 1. Activation scheme of the contrast agent.

We also performed modification of MagSense nanoparticles that have larger diameter in comparison to Miltenyi SPIO (500 nm per DLS measurements). Modification ratio was also about 10^5 GdDOTA-NHS groups per particle.

2. Months 7 - 9.

Activation of the probe will be characterized in model cell free systems as well as in cultured cells. Agarose gel phantoms and purified CaD enzyme will be used for cell free studies. Human breast cancer MCF-7 cell line expressing Cathepsin D will be used in MRI experiments with isolated cells.

Visualization of the in vitro release of Gd from Gd-SPIO complexes by the dual MR contrast agent technique was accomplished in a model cell-free system. A mixture of the contrast agent in PBS buffer (40 µL, pH 4.5)

was placed on the 2% agarose gel layer in a plastic PCR tube. Quantitative T1 maps of the phantoms were acquired on a dedicated small animal MR scanner at 9.4T. Briefly, fast spin echo sequence with RARE factor of 8 was applied with different relaxation delays in the range from 100 ms to 8s and the quantitative T1 map was reconstructed from partly relaxed images using simple exponential fitting *[M = M0•(1 – exp(-t/T1)].* Pre-activation images were taken up to 2h after addition of the agent. No diffusion of Gd-SPIO complexes into the agarose layer was detected at this point (Figure 2b). The agent was activated with recombinant CaD (0.18 units in 25 µl) added to the upper chamber and after 2h incubation, the released GdDOTA molecules diffused into the agarose layer as shown in image (Figure 2c).

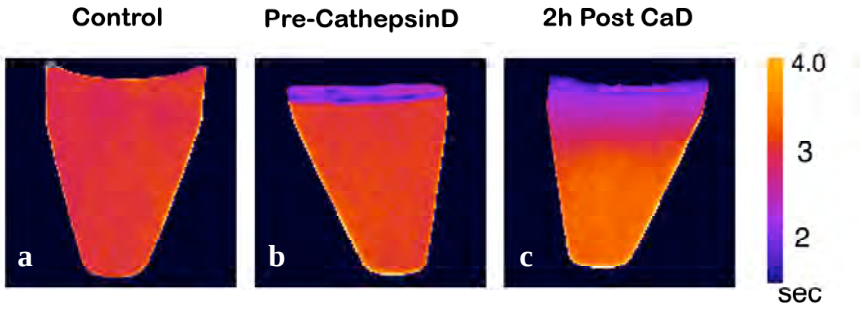

Figure 2. Quantitative T1 maps of the cell-free phantom. No diffusion of the contrast agent was detected in the control sample (no agent was added) (a), and with the agent in the absence of the activating Cathepsin D enzyme (B). After addition of CaD a rapid degradation of the probe and diffusion of the released low molecular weight GdDOTA groups into the gel layer was detected (C).

We also performed studies with cultured MCF-7 cells however only minimal activation of the enzyme was detected possibly because of inhibiting action of the serum present in the medium. We currently are working with serum-free experimental conditions however this issue needs to be addressed for *in vivo* applications of the method. Alternatively, more aggressive MDA-MB-231 cell line will be tested in the model in vitro system with the current cleavable peptide.

3. Months 10-12.

In vivo MRI studies for activation of the contrast agent will be performed with 15 SCID mice inoculated with MCF-7 tumor xenografts. Probes prepared with the specific and scrambled peptide linkers will be used for in vivo studies.

As a proof of principle studies we performed preliminary experiments in vivo with an alternative form of the activated agent, cationic liposomes, loaded with a combination of SPIO (Feridex) and GdDTPA contrast agents. Following the similar strategy, after opening the liposomes, the small molecular weight gadolinium agent will diffuse at significantly larger distance in comparison to massive SPIO nanoparticles. To confirm our concept of dual MR contrast technique and to simulate diffusion properties of the tissue, we constructed a liposome model system. Briefly, liposomes were prepared from DSPC, DSPE-PEG, and cholesterol by the sonication method, followed by the extrusion method. The encapsulating medium contained 50 mL of 200 mM SPIO suspension and 100 mL of 50 mM GdDTPA-BMA in 1 mL of PBS. The liposomes were annealed in the dark at room temperature overnight, followed by extrusion through 200 nm and 100 nm polycarbonate membranes (Avestin Inc., Ottawa, Canada). As controls, liposomes encapsulating either GdDTPA-BMA alone (Lip-Gd) or SPIO alone (Lip-Fe) were also prepared. Free SPIO particles (about 50 nm diameter) were removed from 100-150 nm liposomes by initial centrifugation at 500xg followed by Sepharose® CL-2B column separation using PBS as the mobile phase. Free GdDTPA-BMA was removed by running the sample through a Sephadex® G-50 column in the same manner as the liposomes encapsulating SPIO particles.

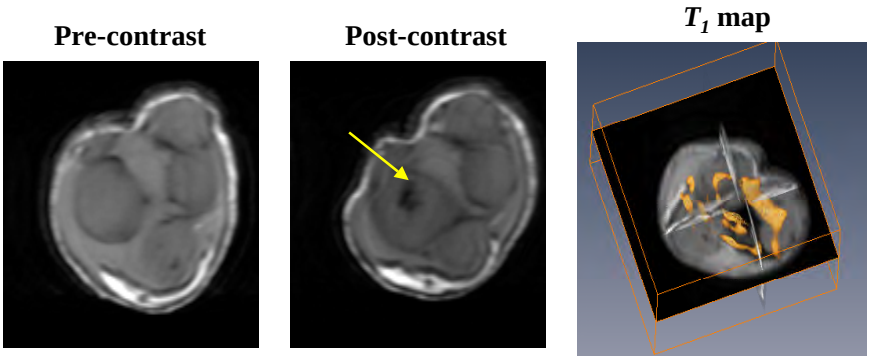

Figure 3. MR images of MatLyLu prostate cancer xenograft acquired before and 2 hr after intratumoral administration of Lip-Gd/Fe suspension (10 mL) at a dose of 14.2 nmol equivalent to Gd. Post-contrast images were co-registered to the pre-contrast images for comparison. Negative contrast in the tumor is generated by extravasated liposomes (yellow arrow). Orange areas on the right image represent leakage of low molecular weight Gd-based contrast agent released from spontaneously disrupted liposomes.

Key research Accomplishments

- Synthesis and characterization of the activated MR contrast agent sensitive to the activity of Cathepsin D enzyme
- Demonstration of the activation of the agent in cell free system by recombinant cathepsin D enzyme
- Proof of the concept studies for detection of the dual SPIO-Gd contrast agent in vivo in preclinical tumor models.

Reportable Outcome

1. Y. Kato, A. Pathak, and D. Artemov "Activated MR Contrast Agent by A Dual Contrast Technique And Their Application", 16th Scientific Meeting of the International Society for Magnetic Resonance in Medicine, Toronto, 2008.

2. D. Artemov and Y. Kato "Molecular MR Imaging of Protease Activity in Breast Cancer with Activated Contrast Agents", 5th Era of Hope Meeting, Baltimore, 2008.

Conclusion

We demonstrated that novel dual contrast agents composed of large negative SPIO-based and positive small molecular weight Gd-based units can be specifically activated by enzymatic activity of the Cathepsin D proteolytic enzyme that is important marker for aggressiveness and invasiveness in breast cancer. The system currently works in cell free system however to improve activation kinetic of the agent by live breast cancer cells the cleavable peptide linker needs to be optimized. Also different breast cancer cell lines with increased invasive behavior could be used in initial experiments. Emergence of the positive contrast by decomposition of the dual MR contrast agents-loaded carrier using liposome model system supports our concept behind in vivo applications of the activated MR contrast agent. However, additional experiments are required to demonstrate robust activation of the agent in vivo in preclinical models of breast cancer.
These findings can facilitate noninvasive MR imaging of breast cancers overexpressing proteolytic enzymes such as Cathepsin D that are implicated in tumor invasion, angiogenesis, and metastasis.

References

Appendices

Please see attached ISMRM abstract.

Supporting Data

Included in the Body of the report.

Activated MR Contrast Agent by A Dual Contrast Technique And Their Application

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Introduction: Gadolinium (Gd)-based contrast agents represent positive contrast agents while superparamagnetic iron oxide (SPIO) nanoparticles are categorized as negative contrast agents. Both types of agents have been used extensively as a single agent, and concomitant use has also been tried pre-clinically (1) and clinically (2) to improve the contrast in diagnostic MR images. The strategy of the activated MR contrast agent developed here is based on the encapsulation of both a positive and a negative contrast agent within the same carrier and subsequent decomposition of the carrier. The concept behind this strategy is that strong negative signal enhancement due to the T_2/T_2* effects of iron oxides dominates the positive T_1 contrast generated by a Gd-based contrast agent when these agents are in close proximity, such as within an intact nanocarrier encapsulating GdDTPA/SPIO, and positive T_1 contrast becomes evident upon release of Gd-based contrast agent from the carrier once the distance between Gd-based contrast agents and SPIO molecules is beyond the T_2/T_2* enhancement range, as illustrated in Fig. 1. One of the suitable applications for the activated contrast agent is noninvasive release monitoring of small molecular weight cargo molecules, GdDTPA, from the carrier and subsequent intratumoral distribution of GdDTPA. Activation of MR contrast enhancement also involves the diffusion of a low molecular weight Gd-based contrast agent from the areas of negative signal enhancement generated by massive SPIO nanoparticles upon degradation of the carrier. This phenomenon is based on a significant restriction of the free diffusion of massive SPIO nanoparticles due to their large sizes (40-70 nm), which results in a significantly shorter diffusion range. Therefore, the low molecular weight Gd-based MR contrast agent can be used as a surrogate marker for other small molecule anticancer agents, assuming their diffusion rates are similar, on the grounds that intratumoral delivery of larger molecules is restricted due to short diffusion distance from vascular surface compared to smaller molecules (3). In this study, we sought the feasibility of the activated MR contrast agent using a dual contrast technique *in vitro* phantom study and *in vivo* mice xenografted tumor models.

Methods: Liposomes were used for a model carrier system. Omniscan® (GdDTPA-BMA) and Feridex® (SPIO)-loaded liposomes (Lip-Gd/Fe) were prepared by the sonication method, followed by extrusion through polycarbonate membrane (pore size: 200 nm first, then 100 nm). Empty liposomes (Lip) and single contrast agent-loaded liposomes (Lip-Gd and Lip-Fe) were also prepared for controls. A dynamic laser-light scattering (DLS) was used for the measurement of particle size, size distribution, and ζ-potential of the resultant liposomes. Gd contents were determined by T_1 relaxation time measurement using MRI, after destruction of liposomes. The encapsulation of SPIO nanoparticles in liposomes was confirmed using atomic force microscope (AFM). *In vitro* visualization study was performed using 2% agarose gel as illustrated in Fig. 1. A multislice-multiecho pulse sequence wi th an echo time (TE) of 15 ms, and six different repetition times (TR) were used on a Bruker 9.4T spectrometer. *In vivo* release monitoring of GdDTPA-BMA from liposomes and subsequent intratumoral distribution were investigated using two xenografted cancer models, a rat prostate cancer MatLyLu and a human breast carcinoma MCF-7 in mice. To obtain both released GdDTPA-BMA and SPIO distribution images, a 3D fast spin echo (RARE: Rapid Acquisition with Relaxation Enhancement) sequence with an effective TE of 50 ms and a TR of 1000 ms was acquired before and after intratumoral or intravenous administration of Lip-Gd/Fe. For the quantitative T_1 map, a 3D RARE pulse sequence with a TE of 50 ms, and five different TRs was acquired with a spatial resolution of 0.125×0.250×0.250 mm. For T_2* acquisition, a 3D fast low-angle shot (FLASH) pulse sequence was used with the following parameters: TE/TR = 7/100 ms; NA = 4. The distribution of the SPIO relative to the tumor vasculature was also visualized *ex vivo* 48 hrs post-i.v. administration of Lip-Gd/Fe in MatLyLu tumor xenograft.

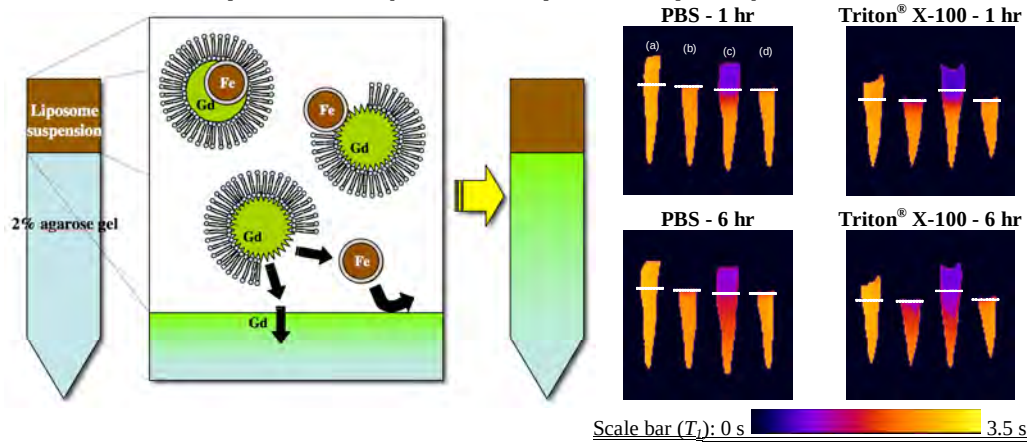

Figure 1. Schematics of *in vitro* release visualization of liposomal dual contrast agents (left panel). (a) Lip, (b) Lip-Gd/Fe, (c) Lip-Gd, (d) Lip-Fe. Quantitative T_1 maps of liposome phantoms are shown in the right panels. White broken lines represent the interface of the suspension/agarose gel layer. Parameters: 3 sagittal slices (st = 1 mm); FOV = 40 × 22 mm; matrix size = 128 × 80; TE = 15 ms; NA = 2.

Results/Discussion: *In vitro* release of MR contrast agent from liposomes with or without Triton® X-100 was successfully monitored using 2% agarose gel system. Intratumoral release and distribution of GdDTPA-BMA was noninvasively monitored *in vivo* following both intratumoral and intravenous administration of Lip-Gd/Fe at a dose of 14.2 nmol eq. GdDTPA-BMA, and 0.43 μmol eq. GdDTPA-BMA, respectively. A restricted SPIO diffusion was also proved by *ex vivo* fluorescent iron staining.

Conclusion: A dual MR contrast technique could be a promising strategy for activated MR contrast agent. Non-invasive release monitoring and subsequent intratumoral distribution of cargo molecules is one of the potential applications for an activated MR contrast agent using a dual contrast technique.

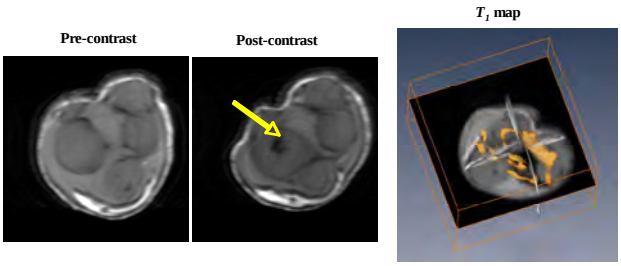

Figure 2. MR images of MatLyLu prostate cancer xenograft before and 2 hr post-intratumoral administration of Lip-Gd/Fe suspension. Post-contrast images were co-registered to the pre-contrast images for comparison. Negative contrast in the tumor is generated by extravasated liposomes (yellow arrow). Orange areas on the right image represent leaking of low molecular weight GdDTPA-BMA released from spontaneously disrupted liposomes.

References: (1) Weissleder R. et al. *AJR Am J Roentgenol* 150, 561 (1988); (2) Suto Y. & Shimatani Y. Br J Radiol 68, 116 (1995); (3) Dreher M.R. et al. *J Natl Cancer Inst* 98, 335 (2006).

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